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RETROVIRUS AND HERPESVIRUS ASSOCIATIONS WITH FIBROPAPILLOMATOSIS OF MARINE TURTLES

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PREFACE

This report is the result of research conducted by Dr. James Casey and colleagues with partial funding support and collaboration from the Southwest Fisheries Science Center Honolulu Laboratory, National Marine Fisheries Service.

The cause of fibropapillomatosis, a debilitating and often fatal tumor disease, remains unknown. However, a multifactoral viral etiology has been implicated, as suggested by Dr. Casey's research reported herein and the work of others. Fibropapillomatosis represents a potentially serious threat to the long-term recovery and healthy maintenance of green turtle populations at several locations worldwide, including Hawaii, Australia, Florida, and areas of the Caribbean. In addition, the disease is known to occur in the loggerhead turtle, *Caretta caretta*, and has recently been histologically confirmed in Pacific populations of the olive ridley turtle, *Lepidochelys olivacea*.

Because this report was prepared by an independent investigator, the statements, findings, conclusions, and recommendations do not necessarily reflect the views of the National Marine Fisheries Service, NOAA, U.S. Department of Commerce.

George H. Balazs
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June 1998

INTRODUCTION

An infectious agent is strongly implicated in the etiology of marine turtle fibropapillomatosis (FP) (Herbst et al., 1995; Herbst et al., 1996; Jacobson et al., 1991). Outbreaks of the disease occurred in captive-reared turtles after contact with wild-caught green turtles, *Chelonia mydas*, with FP (Herbst, 1994; Hoffman and Wells, 1991; Jacobson et al., 1989) and, recently, FP has been experimentally transmitted to green turtles using filtered cell-free tumor homogenates (Herbst et al., 1995; Herbst et al., 1996). These studies, and observations by others (Casey et al., 1997; Jacobson et al., 1991) suggest a virus is involved in the pathogenesis of this disease. However, attempts to isolate any virus in cell culture have been unsuccessful to date.

In this period we have initiated two independent research initiatives to investigate the etiology of FP of green turtles. The first approach is based upon our earlier studies which showed reverse transcriptase activity in many fibropapillomas and fibromas. To further investigate the retroviral origin of this activity, we have employed the Polymerase Chain Reaction (PCR) with degenerate primers targeted to amplify regions of the highly conserved reverse transcriptase polymerase gene and have cloned and sequenced a resulting amplicon. The second approach employs a similar PCR-based approach to amplify, clone, and sequence regions of the conserved herpesvirus DNA polymerase genes in an effort to implicate this class of infectious agent in the genesis of FP.

MATERIALS AND METHODS

Virus Purification

Unless otherwise stated, all operations were performed at 4°C. In brief, 1 g of turtle tissue, stored at -70°C, was minced and dounce-homogenized in 10 volumes g^{-1} of TNES (10 mM Tris-HCl pH 7.5, 1 mM EDTA, 100 mM NaCl, 5% sucrose). The homogenate was centrifuged at 10,000 x g for 20 min. The supernatant was centrifuged at 100,000 x g for 1.5 h in a SW 28 rotor (Beckman Industries). The pellet was resuspended in 1 ml of TNES and layered on a discontinuous sucrose gradient of equal volumes at 15, 35, and 50% TNE sucrose. The gradients were centrifuged for 2 h at 100,000 x g and the 35% to 50% interface collected, diluted tenfold with TNE, and pelleted at 100,000 x g for 1.5 h. The pellet was then resuspended in 100 μl of TNES with 6 mM dithiothreitol and stored at -70°C. Equal aliquotes of these preparations from the same amount of starting material were employed in either the PERT or conventional RT assays to allow

for comparison. For gradient analysis, the initial 100,000 x g pellet from 10 g of tumor was resuspended in 1 ml of TNE, layered on a 15% to 60% continuous sucrose gradient, and centrifuged for 16 h at 100,000 x g.

Tissues and DNA Isolation

Tissues were obtained from 10 Hawaiian green turtles, two Florida green turtles, two Florida loggerhead turtles, *Caretta caretta*, (tumors from loggerhead turtles were provided by Barbara Schroeder, National Marine Fisheries Service, Silver Spring, Maryland), six olive ridley (*Lepidochelys olivacea*), leatherback (*Dermochelys coriacea*), and Kemps ridley turtles (*Lepidochelys kempii*). (Leatherback and Kemps ridley samples were kindly provided by Peter Dutton, National Marine Fisheries Service, La Jolla, California.) Biopsies of normal skin were collected from captive-reared green turtles from Sea Life Park Hawaii, and from clinically tumor-free wild caught Hawaiian green turtles. A green turtle embryo fibroblast cell line, GTEF (Moore et al., 1998), was provided by Melody Moore (U.S. Geological Survey National Wildlife Health Laboratory, Madison, Wisconsin). Tissues were homogenized in TE (10 mM Tris-HCl, pH 8.0, 50 mM EDTA, pH 8.0), and 100 μg/ml of proteinase K and 0.1% sodium dodecyl sulfate (SDS) were added. After overnight incubation at 37°C, the cellular DNA was extracted three times with phenol:chloroform:isoamyl alcohol (25:24:1), two times with chloroform:isoamyl alcohol, ethanol precipitated, resuspended in H_2O, and quantitated.

Consensus Reverse Transcriptase RT-PCR Amplification

To amplify a segment of the pol genes from the retroviruses present in fibropapilloma lesions, PCR was done using degenerate pol primers. These primers encode the amino acid sequences, LPQG (5'-ctcggatccGTNYTNCCNCARGG-3') and YMDD (5'-ctcgtcgacRTCRTCCATR TA-3'), and generate an amplicon of approximately 135 bp (lower case letters represent added restriction sites) from retroviral pol templates. cDNA was prepared from sucrose gradient purified virus. The cDNA was subjected to PCR amplification using the LPQG and YMDD primers. The products were digested with BamHI and SaII and cloned into pBluescript SK- (Stratagene). Clones were sequenced with Sequenase version 2.0 (U.S. Biochemicals) using T3 and T7 primers on double-stranded DNA templates.

Consensus Herpesvirus PCR Amplification

Amplification of a portion of the herpesviral DNA polymerase gene was performed with nested primers targeted to highly conserved amino acid motifs, and modified as previously described

(VanDevanter et al., 1996). One μg of DNA was amplified in the nested PCR reaction. The degenerate primers DFA, ILK, and KG1 were used in the first round PCR. One μl of the first round PCR was used as the template for the second round nested PCR with the TGV and IYG degenerate primers. Automated sequencing with the TGV and IYG primers was done with an ABI 373A automated sequencer (Applied Biosystems, Inc., Foster City, California) at the Biotechnology Resource Center at Cornell University.

Additional sequences were obtained by primary amplification with the consensus primers DFA and KG1 as described above, but with a total of 35 cycles. One μl of the first round PCR reaction was used as the template for the secondary amplification with the DFA upstream primer and a turtle herpesvirus downstream specific primer, GTHV2. The PCR products were purified and directly sequenced with the GTHVPR2 primer which is located internal to the downstream GTHV2 primer.

Specific Turtle Herpesvirus PCR Amplification and Analysis of PCR Products

One μg of DNA isolated from various tissues was subjected to PCR amplification with turtle-specific herpesvirus primers, GTHV1 and GTHV2. Fifteen μl of each PCR amplification reaction were separated on a 2% agarose gel, transferred to nitrocellulose, and hybridized to a ^{32}P-labeled turtle-specific probe (GTHVpol). The GTHVpol probe was amplified by PCR from a gel-purified fragment corresponding to the turtle DNA polymerase region from the pGTHVpol plasmid clone using a random primed DNA labeling kit (Boehringer Mannheim, Indianapolis, Indiana) according to manufacturer's instructions. The GTHVPR1 and GTHVPR2 primers are located internal to the turtle herpesvirus specific primers, GTHV1 and GTHV2. The sensitivity of the turtle-specific PCR assay using GTHV1 and GTHV2 primers was tested on serial 10-fold dilutions of pGTHVpol in 1 μg of control green turtle DNA (GTEF cell line)(Moore et al., 1998).

Phylogenetic Analysis

Phylogenetic trees were generated with the phylogeny inference package (PHYLIP) (Felsenstein, 1995) based on a CLUSTAL multiple sequence alignment (DNAStar Inc., Madison, Wisconsin) of herpesvirus DNA polymerase gene sequences. The phylogenetic tree in Figure 1 was generated by the principle of maximum parsimony (PROTPARS). Trees were also constructed based on distance matrices obtained by the maximum likelihood approach and unweighted pair group method by arithmetic averaging (UPGMA) and neighbor joining (NEIGHBOR) methods.

Development of Retroviral Nucleic Acid Probes Specific to Fibropapillomatosis of Hawaiian Green Turtles

RT-PCR Analysis for Retroviral Pol Sequences

The two regions assessed for primer targeting are YMDDI and LPQG. They are separated by approximately 120 bases and generate the correct sized amplicons when challenged with control retroviral virion RNA. Walleye dermal sarcoma virus was used as a positive control in this experiment. We have amplified a sequence of the predicted size from one fibropapilloma RNA preparation while we have failed to amplify additional retroviral sequences from RNA from four additional fibropapillomas. The one positive sample was cloned into pBluescript (SK-), sequenced and compared to retroviral sequences logged into GenBank. Interestingly, the green turtle retroviral sequence showed the highest similarity to the retroviruses associated with pulmonary carcinoma of sheep, termed "jaagsiekte sheep retrovirus" and a newly described exogenous human retrovirus (HRV5).

Southern Blot Analysis of Turtle DNAs

Southern blot analysis using the purified clone insert, labeled by PCR amplification, showed conclusively that the green turtle pol sequence, like jaagsiekte in sheep, is endogenous to this species and present at a level of approximately 10 copies per cell. DNAs from loggerhead, leatherback, olive ridley, kemps ridley, and green turtle were digested with BamH1 and hybridized. The results suggest that the leatherback is lacking most of the copies of this jaagsiekte-like sequence if not totally devoid of this gene indicating an early divergence of this species. Furthermore, the ridleys share a minor polymorphism which suggests that they are closely linked in evolutionary lineage.

Northern Blot Analysis of RNAs from Fibropapillomas

The single Molecular clone that was scored as a consensus pol gene was labeled and used as a hybridization probe in a Northern blot analysis of RNAs from green turtle fibropapillomatosis (GTFP) samples. Total RNAs from uninvolved normal skin samples were also analyzed and compared with RNA from tumor samples to assess for the specific expression of this cloned sequence. No hybridization specific to the GTFP samples was obtained. We conclude that the retroviral pol clone is endogenous and likely not specifically involved in the induction of GTFP.

Herpesvirus Etiology of Green Turtle Fibropapillomatosis

Amplification of a Herpesvirus DNA Polymerase Sequence

To identify and characterize a GTFP-associated herpesvirus, we used a nested PCR assay that employs degenerate primers. Amplification products of 483 bp were detected and directly sequenced from fibropapillomas from Hawaiian and Florida green turtles. A phylogenetic tree was constructed using the PROTPARS program of the PHYLIP package from the amino acid sequences contained in this conserved region of the DNA polymerase gene of several herpesviruses (Fig. 1). Trees with similar branching patterns were generated with the neighbor joining programs of the PHYLIP package (data not shown). This analysis placed the turtle herpesviruses in the *Alphaherpesvirinae* subfamily.

Detection of Herpesvirus in Fibropapillomas from Loggerhead and Olive Ridley Turtles

To determine if fibropapillomas from loggerhead and olive ridley turtles contain a herpesvirus, DNA extracted from six tumors (two loggerhead and four olive ridley tumors) was subjected to PCR using a nested PCR assay. The sequence of the fragment of the herpesvirus DNA polymerase gene from the two loggerhead turtles was identical to the sequence obtained from the Florida green turtles. All of the sequences obtained from the four olive ridley tumors were identical, and differed from the Florida and Hawaii green turtle herpesviral DNA sequences by 3%. DNA isolated from five samples of normal tissue (heart, kidney, skin, and spleen) from two olive ridley turtles was subjected to the nested PCR assay using degenerate primers, and all of the samples were found to be negative (Table 1).

Tissue Distribution of Green Turtle Herpesvirus

To specifically amplify a region of the green turtle herpesvirus DNA polymerase gene, defined, non-degenerate primers were designed based on the sequence obtained. The turtle herpesvirus-specific primers, GTHV1 and GTHV2, are located internal to the degenerate primer sites and amplify a 165 bp product. These primers allowed detection of femtogram quantities of cloned target DNA, pGTHV, diluted in 1 μg of control cellular DNA (data not shown). DNA was prepared from fibropapillomas collected from 10 Hawaiian and two Florida turtles, and subjected to PCR using the GTHV1 and GTHV2 primers. Often, several fibropapillomas were present on the turtles, therefore, we isolated DNA from two (five turtles) or three (two turtles) individual tumors obtained from Hawaiian turtles and performed

PCR with the turtle herpesvirus-specific primers. All of the fibropapillomas (n = 23) contained the turtle herpesvirus sequence (Table 1). In addition, three of the Hawaiian turtles had tumors in visceral organs (heart, lung, liver, and kidney), all of which contained viral DNA (Table 1). DNA isolated from skin biopsies from captive-reared and wild-caught green turtles were negative by PCR for the turtle herpesvirus sequence (Table 1).

To determine the tissue distribution of GTHV, PCR was performed on DNA extracted from several different organs from Hawaiian and Florida turtles using the GTHV1 and GTHV2 primers (Fig. 2, Table 1). On gross examination of each tissue there was no evidence of tumor. No viral sequences were detected in blood, brain, liver, or spleen. However, GTHV DNA sequences were detected from DNA isolated from the lung of two of four Hawaiian turtles and from the heart and kidney from one of the Florida turtles. Herpesviral DNA was detected by PCR in three of four gonad samples. Four of seven normal skin samples collected from tumor-positive Hawaiian turtles were also found to contain viral DNA by PCR.

DISCUSSION

The first stage of this study represents an effort to extend the data that implicate a retrovirus involvement in the etiology of FP. The data clearly show that a retroviral pol-related sequence has been isolated and that this sequence is endogenous to a number of marine turtles. However, Northern blot analysis shows that this sequence is not generally expressed in fibropapillomas at a level detected by this technique, presumably one to five copies per cell. Since the retroviral pol clone was obtained by RT-PCR, the implication that some low level expression exist in these tumors is apparent. The contribution of this sequence and its presumably associated retroviral genes to tumorigenesis remains to be determined.

In the second stage of this study we used a nested PCR assay with degenerate primers, designed to amplify a region of the DNA polymerase gene of herpesviruses that contains highly conserved amino acid motifs (Rose et al., 1997; VanDevanter et al., 1996), to identify herpesvirus sequences that are etiologically associated with FP of marine turtles. This sensitive PCR assay has been used to amplify sequences of several known herpesviruses (VanDevanter et al., 1996), and more recently was employed to describe two newly identified herpesviruses associated with simian retroperitoneal fibromatosis (Rose et al., 1997). Turtle herpesvirus sequences are detected in all fibropapillomas suggesting the presence of newly identified turtle herpesviruses that are associated with these tumors. Phylogenetic analysis indicates that, like HSV, BHV-1, PRV, and Gallid HV, these

viruses belong to the *Alphaherpesvirinae* subfamily. However, final classification and the relationship among the alphaherpesviruses awaits further sequence information.

The turtle herpesvirus sequence was detected in all tested fibropapillomas and fibromas. The presence of low levels of turtle herpesviral DNA, detected only by PCR, in some of the apparently normal lung, heart, kidney, and skin samples was not surprising, as tumors often develop in these organs and skin is the primary target tissue for tumor development. It is possible that very small tumors were present that were not visible grossly. Detection by PCR of viral DNA in some of the gonad samples from Hawaiian turtles was unexpected, as tumors have not previously been seen in this tissue (T.M. Work, unpublished data).

We also identified a herpesvirus DNA polymerase sequence in two fibropapillomas from loggerhead turtles and in four tumors from olive ridley turtles. The sequence of the 483 bp fragment of the herpesvirus DNA polymerase gene from olive ridley turtles allowed placement of these amplicons in the phylogenic tree. These results indicate that the Florida and Hawaiian green turtles, and the olive ridley are distinct, but, closely related herpesviruses.

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CITATIONS

Casey, R. N., S. L. Quackenbush, T. M. Work, G. H. Balazs, P. R. Bowser, and J. W. Casey.
1997. Evidence for retrovirus infections in green turtles *Chelonia mydas* from the Hawaiian Islands. Dis. Aquat. Org. 31:1-7.

Felsenstein, J.
1995. PHYLIP (Phylogeny Inference Package) Version 3.57c, University of Washington, Seattle.

Herbst, L. H.
1994. Fibropapillomatosis of marine turtles. Annu. Rev. Fish. Dis. 4:389-425.

Herbst, L. H., R. Moretti, T. Brown, and P. A. Klein.
1996. Sensitivity of the transmissible green turtle fibropapillomatosis agent to chloroform and ultracentrifugation conditions. Dis. Aquat. Org. 25:225-228.

Herbst, L. H., E. R. Jacobson, R. Moretti, T. Brown, J. P. Sundberg, and P. A. Klein.
1995. Experimental transmission of green turtle fibropapillomatosis using cell-free tumor extracts. Dis. Aquat. Org. 22:1-12.

Hoffman, W., and P. Wells.
1991. Analysis of a fibropapilloma outbreak in captivity. *In* M. Salmon and J. Wyneken (comps.), Proceedings of the Eleventh Annual Workshop on Sea Turtle Biology and Conservation, February 26-March 2, 1991, Jeckyll Island, Georgia, p. 58-68. U.S. Dep. Commer., NOAA Tech. Memo., NMFS-SEFSC-302.

Jacobson, E. R., C. Buergelt, B. Williams, and R. K. Harris.
1991. Herpesvirus in cutaneous fibropapillomas of the green turtle *Chelonia mydas*. Dis. Aquat. Org. 12:1-6.

Jacobson, E. R., J. L. Mansell, J. P. Sundberg, L. Hajjar, M. E. Reichmann, L. M. Ehrhart, M. Walsh, and F. Murru.
1989. Cutaneous fibropapillomas of green turtles (*Chelonia* mydas). J. Comp. Pathol. 101:39-52.

Moore, M. K., T. M. Work, G. H. Balazs, and D. E. Docherty.
1998. Preparation, cryopreservation, and growth of cells prepared from the green turtle (*Chelonia mydas*). Methods in Cell Science 19:161-168.

Rose, T. M., K. B. Strand, E. R. Schultz, G. W. R. Schaefer, Jr., M. E. Thouless, C. C. Tsai, and M. L. Bosch.
1997. Identification of two homologs of the kaposi's sarcoma-associated herpesvirus (human herpesvirus 8) in retroperitoneal fibromatosis of different macaque species. J. Virol. 71:4138-4144.

VanDevanter, D. R., P. Warrener, L. Bennett, E. R. Schultz, S. Coulter, R. L. Garber, and T. M. Rose.
1996. Detection and analysis of diverse herpesviral species by consensus primer PCR. J. Clin. Microbiol. 34:1666-1671.

Table 1.--Detection of turtle herpesvirus in tissues.

	n	No. positive (%)
Tumor animals	18[a]	
fibropapilloma	29	29(100)
fibroma	5	5(100)
blood	3	0(0)
brain	3	0(0)
gonad	4	3(75)
heart	6	1(17)
kidney	5	1(20)
liver	5	0(0)
lung	4	2(50)
skin	7	4(57)
spleen	3	0(0)
Non-tumor animals	16[b]	
skin	16	0(0)
kidney	1	0(0)
heart	1	0(0)
spleen	1	0(0)

[a] 10 Hawaiian green turtles, 2 Florida green turtles, 2 loggerhead turtles, and 4 olive ridley turtles

[b] 14 Hawaiian green turtles and 2 olive ridley turtles

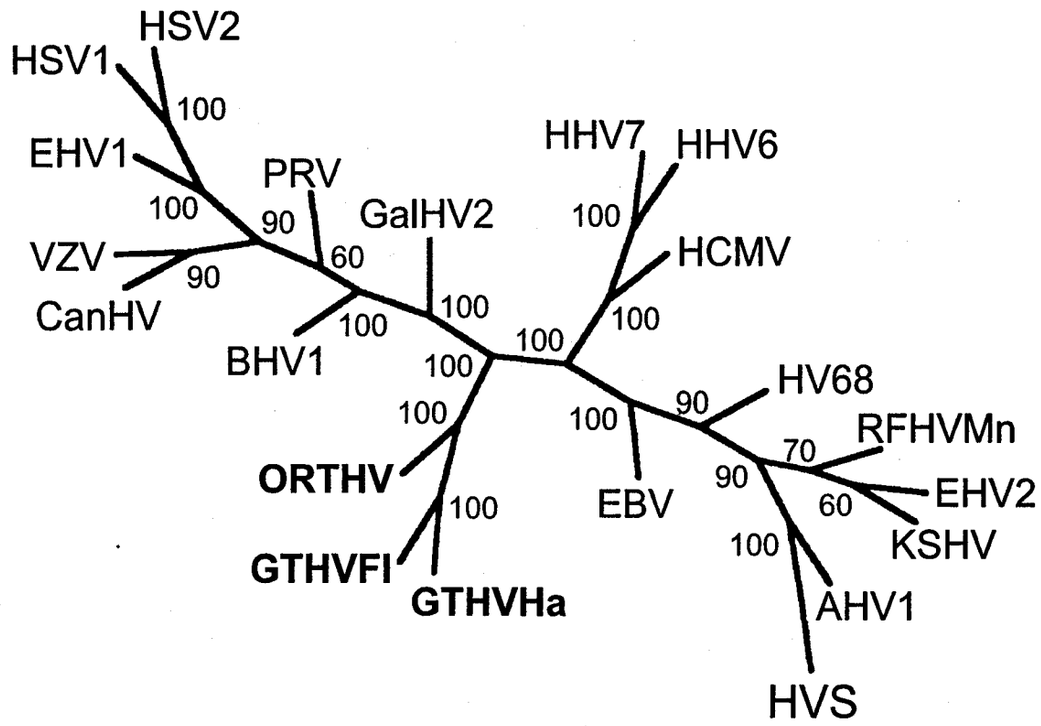

Figure 1.--Phylogenetic analysis of the turtle herpesvirus sequences (GTHV-Ha, GTHV-Fl, and ORTHV) and the corresponding amino acid region from the DNA polymerase genes of other known herpesviruses. The unrooted phylogenetic tree was generated with the PHYLIP package. The numbers at each branch indicate the percent frequency of this grouping after 100 bootstrap evaluations. AHV-1 (alcelaphine herpesvirus 1), CanHV (canine herpesvirus 1), EHV-2 (equine herpesvirus 2), HV68 (murine gammaherpesvirus 68), HSV-2 (HHV-2), HVS (Saimirine herpesvirus 2), and RFHVMn (retroperitoneal fibromatosis herpesvirus *Macaca nemestrina*;), BHV-1, EHV-1 (equine herpesvirus 1, GalHV-2, HSV-1, PRV, VZV, HHV-3, HCMV (human cytomegalo virus), HHV-5, HHV-6, HHV-7, EBV, and KSHV.

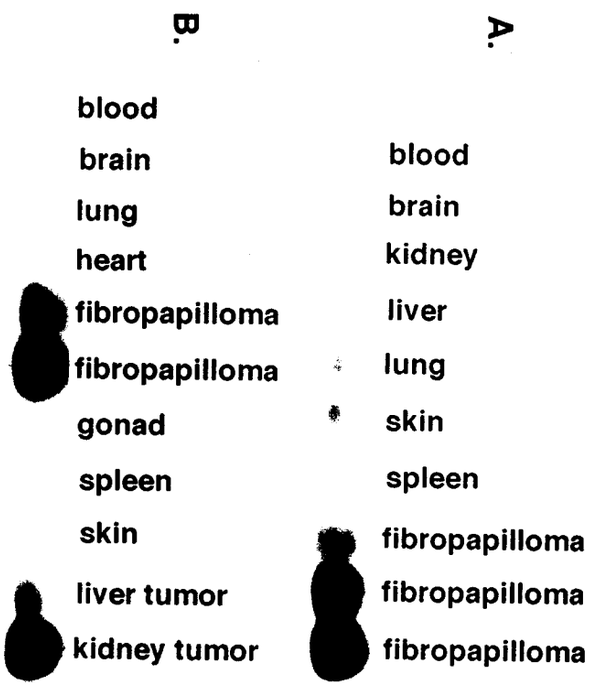

Figure 2.--Southern blot analysis of PCR products from turtle 12428 (A) and turtle 390 (B). One μg of DNA isolated from various tissues was subjected to PCR amplification with turtle-specific herpesvirus primers (GTHV1 and GTHV2). Amplification products were separated by electrophoresis, transferred to nitrocellulose, and hybridized with a ^{32}P-labeled, turtle herpesvirus-specific probe (GTHVpol).